SUPPLEMENTAL MATERIAL

Inhibition of mTOR signaling enhances maturation of cardiomyocytes derived from human induced pluripotent stem cells via p53-induced quiescence

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SUPPLEMENTAL METHODS

Cell lines

Three human iPSC lines were used in this study. The BJ-RiPS cell line (male donor, fibroblast derived) was obtained from the Harvard Stem Cell Institute (HSCI) Induced Pluripotent Stem Cell (iPSC) Core facility. The UCSD142i-86-1 iPSC line (female donor, fibroblast-derived) was generated by Dr. Kelly Frazer's laboratory at the University of California San Diego and was distributed by WiCell. The commercially-available Gibco episomal-derived iPSC line (CD34+ cord blood mononuclear cell-derived) was obtained from ThermoFisher Scientific. Overall passage numbers for each cell line were less than 70, and new lower passage vials were thawed when passage numbers approached 20 since last thawing. Cells were maintained in StemFlex (ThermoFisher) with passage every 3-4 days using ROCK inhibitor for 24 hours after splitting. Representative data depicted in the figures are from individual experiments with a single cell line; the cell line used for each figure is indicated in the figure legend (abbreviated by BJRIPS-CMs, Gibco iPS-CMs, or UCSD-CMs). Key experiments were performed in all three cell lines to confirm reproducibility.

Differentiation of induced pluripotent stem cell derived-cardiomyocytes

To prepare for differentiation, iPSCs were plated at a density between 20,000 to 80,000 cells (depending on the cell line) per well of a 12-well plate at four days prior to onset of differentiation. Cells were differentiated according to the protocol previously described by Lian et al. with some modifications.[1] Basal media was changed from StemFlex to

RPMI/B27 on day 0 of differentiation. The GSK3 inhibitor, CHIR99021 (6 μM), was added for 48 hours from day 0 to day 2 of differentiation when cells were approximately 70-80% confluent. The Wnt antagonist IWP4 (5 μM) was added from days 2 to 4 of differentiation. Insulin (10 μg/ml) was added to the media starting on day 7 of differentiation, and media was changed every 2-3 days. In general, we observed higher purity (TNNT2+) cardiomyocytes when beating initiated prior to day 10 of differentiation, therefore only batches with onset of beating between days 7-10 of differentiation were included in this study. Beating cardiomyocytes were treated with Torin1 (200 nM, unless otherwise noted) or vehicle (0.02% DMSO) beginning at approximately 2 days after onset of beating for 7 days (media changed with fresh Torin1 or DMSO every 2-3 days), unless otherwise noted. After Torin1 treatment, media was switched back to RPMI/B27/insulin and maintained with media change every 2-3 days until endpoint assay. In a few assays, fatty acids were added in the form of chemically defined lipids (Gibco) diluted 1:100 simultaneous with Torin1 treatment. In some other assays, fetal bovine serum (10% FBS) was added to cells after completion of Torin1 treatment for 2-4 days.

Quantitative reverse transcriptase polymerase chain reaction

To isolate RNA from cells, RiboZol (VWR) was used followed by the E.Z.N.A. Total RNA I kit (Omega) according to the manufacturers' instructions. RNA was reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad) and the primer

pairs listed in Supplemental Table 1 with a Bio-Rad CFX384 Real-Time thermal cycler. TATA-binding protein (TBP) was used as the housekeeping gene.

Western analysis

Cells were lysed with lysis buffer (1% Triton X, 0.35% Na-deoxycholate, 1 mM EDTA, 1% protease inhibitor cocktail (Sigma), 1% phosphatase inhibitor cocktail 2 (Sigma), 1% phosphatase inhibitor cocktail 3 (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF)) and total protein was measured with the Pierce BCA Protein Assay Kit. Samples with equal total protein quantities (prepared in Laemmli Sample Buffer (Bio-Rad)) were loaded into a Mini-Protean TGX Precast Gel (Bio-Rad) and electrophoresed for 1 hour at 100 V before transfer to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The Precision Plus Protein Dual Color Standards molecular weight ladder was used to estimate molecular weight of detected bands (Bio-Rad). Membranes were blocked with 5% milk or bovine serum albumin then incubated with the primary antibody (Supplemental Table 2) overnight at 4 degrees C followed by incubation with the horseradish peroxidase-conjugated secondary antibody (Supplemental Table 2) for 1 hour at room temperature. The Amersham ECL Western Blotting Detection kit was used to detect the protein signal by chemiluminescence, and band densities were quantified using ImageJ software (NIH).

Flow cytometry

Cells were dissociated and $5x10^5$ to $1x10^6$ cells were used per condition. For cell cycle analysis, $5x10^5$ cells per condition were fixed in 70% cold ethanol for at least 2 hours to

overnight at -20 degrees C. Cells were stained first with AlexaFluor 647-TNNT2 in Permeabilization Buffer (Thermo) then with Hoechst 33342 (2 μg/ml) and pyronin Y (4 μg/ml) for 20 minutes to distinguish between cells in G_0, G_1, and S-G_2-M phases. For other stains, cells were fixed in eBioscience Intracellular Fixation buffer for 30 minutes followed washing twice in eBioscience Permeabilization Buffer and stained with the appropriate primary antibody (Supplemental Table 2) in eBioscience FACS Staining buffer for 30-60 minutes. If a secondary antibody was used, cells were incubated with the appropriate secondary antibody (Supplemental Table 2) for 30-60 minutes. The geometric mean fluorescence intensity (MFI) was quantified for TNNT2 and Kir2.1 antibodies. Data were acquired via a BD LSRII instrument and analyzed using FlowJo software.

Immunocytochemistry

Cells were dissociated from 12 well plates after differentiation with 0.1% trypsin-EDTA and replated into Geltrex-coated 4-chamber microscope slides. Cells were fixed in 4% paraformaldehyde then permeabilized with 0.5% Triton X-100. After blocking with 5% BSA, cells were incubated with primary antibodies (Supplemental Table 2) for 30 minutes at room temperature followed by incubation with the secondary antibody (Supplemental Table 2) for 45 minutes at room temperature in the dark. After washing with phosphate buffered saline (PBS), Vectashield mounting agent with DAPI (for slides) or DAPI (for plates) was applied and cells were visualized with a confocal microscope (Zeiss LSM 700). Muscular thin films (MTFs) were stained in a similar

manner except DAPI (1 µg/ml) was applied for 5 minutes before rinsing and transferring MTFs into PBS until imaging.

Imaging quantification of proliferating cardiomyocytes

Cardiomyocytes were differentiated in 12 well plates then fixed and stained in the original plates to minimize potential for selection of cells that survive dissociation. Cells were stained with Ki67/TNNT2/DAPI or phospho-H3/TNNT2/DAPI as described in Immunostaining methods. Images were acquired with a fluorescent microscope. Monolayer regions containing TNNT2+ cells were randomly selected from 5 fields of view in the plate with a 10x objective. The particle analysis feature of ImageJ was used to quantify the number of cells per field of view that were positive for DAPI, Ki67, or pH3. To control for variability across samples, a signal threshold of <500 was set DAPI and pH3 (488 nm) channels and a signal threshold of <600 was set for Ki67 (488 nM). Particle sizes between 10-1000 pixels^2 were included in the analysis. The percentage of Ki67+ cells or pH3+ cells out of the total number of cells marked by DAPI and TNNT2 was quantified. A chi-squared test was used to evaluate for statistical significance.

Muscular thin films

Gelatin muscular thin films (MTFs) were prepared as previously described.[32] Square 22x22 mm glass coverslips were covered with low adhesive tape (Patco 5560 Removable Protective Film Tape), and an Epilog Mini 24 laser engraving instrument was used to cut the tape such that two inner rectangles (3 x 10 mm for cantilever region and 7 x 10 mm for base region) were surrounded by an outer border. The larger inner

rectangle was peeled away and this region was activated with 0.1 M NaOH for 5 minutes, then 0.5% (3-aminopropyl)triethoxysilane (APTES) in 95% ethanol for 5 minutes, then 0.5% glutaraldehyde solution for 30 minutes. The glass coverslips were then rinsed and dried. Next, the tape covering the smaller inner rectangle region was peeled away. A 20% w/v stock gelatin (175g bloom, Type A, Sigma) in distilled water solution was dissolved at 65˚C for 30 minutes and an 8% w/v stock microbial transglutaminase (MTG) (Ajinomoto) in distilled water solution was dissolved at 37˚C for 30 minutes. The stock gelatin and stock MTG solutions were mixed 1:1 immediately prior to use for a final concentration of 10% w/v gelatin and 4% w/v MTG. The solution was pipetted into the exposed square regions on the glass coverslips. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) stamps with 25 μm ridge width x 4 μm groove width line features were placed on the gelatin and a 500 g weight was placed on top of the PDMS stamp. The gelatin was allowed to set overnight at room temperature. The next day, the weight was removed and the glass coverslips with gelatin and PDMS stamps were placed in distilled water for 30 minutes to re-hydrate the gelatin prior to removal of the PDMS stamp. The remaining tape along the border of the gelatin was removed. The MTFs were dabbed dry with a Kimwipe then the cantilevers (1 mm wide x 3 mm long) were cut 6-7 times using an Epilog Mini 24 laser engraver with 3% power, 7% speed, and a frequency of 2000 Hz. MTFs were sterilized in 70% ethanol for 5 minutes followed by exposure to UV light in a biosafety cabinet overnight in PBS. MTFs were coated with Geltrex (Invitrogen) for 30 minutes at room temperature prior to seeding cells. iPSC-derived cardiomyocytes after completion of Torin1 or vehicle treatment were dissociated from 12 well plates and re-seeded onto the MTFs at

a density of 800,000 cells per 100 µL. The cells were allowed to adhere for 1-2 hours in the 100 µL volume at 37˚C then an additional 4 ml of RPMI/B27/insulin + 10% FBS were added to each well of a 6 well plate. The following day, the media was changed back to non-serum containing RPMI/B27/insulin. Cells were allowed to adhere and recover from re-plating for 4-5 days prior to imaging/pacing. On the day of analysis, the gelatin MTFs were transferred to a 35 mm Petri dish containing Tyrode's solution (1.8 mM $CaCl_2$, 5 mM glucose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM MgCl, 5.4 mM KCl, 135 mM NaCl, 0.33 mM of NaH_2PO_4, pH 7.4). Excess gelatin surrounding each cantilever was carefully removed under a dissection microscope (Leica MZ9.5) and the cantilevers were carefully released from the glass using fine forceps. The MTFs were maintained at 37˚C in a heating block during measurements. Two platinum electrodes with 2 cm spacing were placed in the dish and a MyoPacer Cell Stimulator (IonOptix) was used to pace MTFs from 1-4 Hz at 20V. Images were acquired at 20 frames per second using a Basler A601f-2 camera. The radius of curvature for each MTF was quantified using ImageJ. Thickness of the MTF was determined using a Zeiss LSM confocal microscope. The modified Stoney's equation was used to calculate force for each MTF using the radius of curvature, thickness, and elastic modulus.[33] The maximum and average systolic, diastolic, and twitch (systolic – diastolic stress) stresses were calculated for each MTF.

Seahorse Mito Stress Test and Glycolysis Stress Test

The Seahorse XFe96 Analyzer (Agilent) was used to assess metabolic activity of differentiated cardiomyocytes. Seahorse XFe96 cell culture microplates were coated

with Geltrex (1:100 dilution in DMEM:F12). Cardiomyocytes were differentiated as described above then treated with DMSO or Torin1 (200 nM) starting at approximately two days after onset of beating for one week (~days 9-16 of differentiation for most batches). After treatment, all cells were changed back into RPMI/B27/insulin media until assay preparation (performed at or before day 35 of differentiation). One day prior to assay, cardiomyocytes were dissociated with 0.1% trypsin-EDTA for 5 minutes then neutralized with RPMI/B27/insulin + 10% fetal bovine serum (FBS) and plated a density of 20,000-30,000 cells per well. Cardiomyocytes were maintained in serum-containing medium overnight to facilitate cell adhesion. One hour prior to the assay, the plates were changed to Seahorse assay media (XF Base Medium supplemented with 25 mM glucose, 1 mM sodium pyruvate, and 1 mM GlutaMAX), then incubated at 37 degrees C in a CO2-free incubator with Seahorse assay media. The XF Cell Mito Stress Test Kit was used according to the manufacturer's instructions, with the following final concentrations for the injected compounds: 2 μM oligomycin, 2 μM 2-[2-[4-(trifluoromethoxy)phenyl]hydrazinylidene]-propanedinitrile (FCCP), and 0.5 μM rotenone/antimycin A. The XF Glycolysis Stress Test was used according to the manufacturer's instructions, with 10 mM glucose, 1 μM oligomycin, and 50 mM 2-deoxyglucose. Following measurement of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in response to serial injections of the stress test kit compounds, final cell density was determined using a CyQUANT assay (Thermo Fisher Scientific) to account for cell loss during washes. Cardiomyocytes not used for the Seahorse assay but at a similar day of differentiation were used to create a standard curve for the CyQUANT assay to quantify absolute cell number in order to account for

cell loss during washing/mixing steps. OCR values were normalized to absolute cell number determined by the CyQUANT assay. Data were normalized to the average baseline value for each well. Baseline OCR values were calculated by averaging the first three OCR measurements (0-12 minutes) prior to injection of compounds, maximal OCR values were calculated by subtracting the average non-mitochondrial OCR values (OCR following rotenone/actinomycin A) from the OCR values after the injection with the uncoupler FCCP, and respiratory reserve capacity values were calculated by subtracting baseline OCR values from OCR values after addition of FCCP. For the Glycolysis Stress Test, the glycolysis ECAR was calculated by subtracting the average non-glycolytic acidification values (after 2-DG) from the values after glucose administration, the glycolytic capacity was calculated by subtracting the non-glycolytic acidification ECAR values from ECAR values after addition of oligomycin, and the glycolytic reserve was calculated by subtracting the ECAR values after addition of glucose from the ECAR values after addition of oligomycin, according to the manufacturer's instructions.

Voltage analysis with Vala Kinetic Image Cytometer

Following completion of Torin1 treatment, cardiomyocytes were dissociated as described above then replated a density of 20,000-30,000 cells per well in a Geltrex-coated Greiner Cellstar black 96 well plate. FluoVolt dye (1:1000 from stock in the FluoVolt Membrane Potential Kit, Thermo) and Hoechst 33258 stain (20 µg/ml) in RPMI1640 (no phenol red) (supplemented with OxyFluor (Oxyrase, 1:100 dilution), 10 mM sodium lactate) were added to each well 15 minutes prior to assay. Cells were

electrically stimulated with 15 V at a frequency of 0.5 pulses/sec and a pulse width of 5 msec for 5 pulses using the IC200 Kinetic Image Cytometer (Vala). Images were acquired at 20x and at a frame rate of 70 frames per second. Voltage analysis was performed using the CyteSeer image analysis software, which automatically recognizes Hoechst-stained nuclei, segments, and identifies each cell to quantify voltage data for each cell captured in the field of view.[34] Cells were identified by Hoechst-stained nuclei, segmented into individual cells and automatically numbered by CyteSeer software (Figure 4C). Within each cell, fluorescence intensity was recorded from within a circular region within each numbered, nucleated cell. Cells were included if four voltage peaks were detected (first paced beat was only partially captured by the imaging system); cells with fewer than four peaks indicated inadequate capture, and cells with more than four peaks indicated that cells had a spontaneous beating rate that was faster than the paced beats. Data from approximately 300-500 individual cells were included for analysis for each condition. Baseline fluorescence was used to normalize for variability in fluorescence intensity between individual cells. The peak rise time, CTD25 (25% duration of the calcium transient, or duration at 25% decline from maximum amplitude), CTD75 (75% duration of the calcium transient, or duration at 75% decline from maximum amplitude, T75-25 (time for voltage to decay from 75% to 25% of maximum), and downstroke velocity were quantified using the CyteSeer image analysis software.

Calcium transients analysis with Vala Kinetic Image Cytometer

To perform calcium transients analysis, 50ul of 10ug/ml Hoeschst 33258 and 5 μM Fluo-4 AM was added to each well of a 96 well plate and incubated for 10 minutes at 37

degrees C. Media was replaced with RPMI without phenol red supplemented with 1:100 Oxyfluor and 10uM sodium lactate. Isoproterenol (1 μM) was added to the media for isoproterenol experiments. Pacing protocol was 5 seconds delay without pacing followed by 5 seconds pacing at 1Hz and 5 seconds imaging without pacing. Imaging was performed using the Vala Kinetic Image Cytometer at 30 fps with 33.30 ms exposure at 14.25 intensity. Stimulation pulse with was 5 ms with 15V voltage. Data was collected by CyteSeer analysis program.

NanoString analysis

We performed gene expression analysis using NanoString technology which employs an optical counting method of fluorescently barcoded RNA according to the manufacturer's instructions. We selected the nCounter PanCancer Pathways Panel which includes 770 genes from several growth and cell cycle-related pathways, including cell cycle, apoptosis, Wnt, transcriptional regulation, PI3K, and TGF-β. We selected this panel to provide a multiplex analysis of pathways associated with cell cycle regulation, metabolism, quiescence and senescence. We differentiated cardiomyocytes in a 12-well plate then treated cardiomyocytes with Torin1 (200 nM) or vehicle (DMSO) starting ~2 days after onset of beating for 1 week (days 9-16 for this batch). After completion of Torin1 treatment, cells were switched back to maintenance media (RPMI + B27 + insulin). Beginning on day 18 of differentiation, we added 10% fetal bovine serum (FBS) to half of the wells per treatment group and continued FBS treatment for 4 days (until day 22 of differentiation). We harvested cells in RiboZol then extracted total RNA from each well as described above for qPCR preparation. We prepared 200 ng

total RNA then hybridized our samples with the PanCancer Pathways Panel reagents for 16 hours at 65 degrees C according to the nCounter XT Gene Expression Assay. Samples were processed on NanoString prep station then imaged with the nCounter instrument (NanoString Technologies). Data were analyzed using nSolver 4.0 software with the Advanced Analysis Module 2.0 with normalization to internal housekeeping genes including in the panel to perform unsupervised hierarchical clustering, differential expression analyses, and pathway scoring using the R statistical package.

Supplemental Table 1. Primers for qPCR

Gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
ATP2A2 (SERCA2a)	ATGGGGCTCCAACGAGTTAC	TTTCCTGCCATACACCCACAA
CACNA1c	TGATTCCAACGCCACCAATTC	GAGGAGTCCATAGGCCATTACT
CCNA1	GAGGTCCCGATGCTTGTCAG	GTTAGCAGCCCTAGCACTGTC
CCNB1	AATAAGGCGAAGATCAACATGGC	TTTGTTACCAATGTCCCCAAGAG
CCNC	CCTTGCATGGAGGATAGTGAATG	AAGGAGGATACAGTAGGCAAAGA
CCND1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
CDK3	GAAGGTAGAGAAGATCGGAGAGG	GTCCAGCAGTCGGACGATG
CD36	CTTTGGCTTAATGAGACTGGGAC	GCAACAAACATCACCACACCA
CDKN1a (p21)	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCTC
CDKN1b (p27)	AACGTGCGAGTGTCTAACGG	CCCTCTAGGGGTTTGTGATTCT
CDKN2a (p16)	GATCCAGGTGGGTAGAAGGTC	CCCCTGCAAACTTCGTCCT
GATA4	CGGCGAGGAGGAAGGAGCCA	TGGGGGCAGAAGACGGAGGG
HES1	TCAACACGACACCGGATAAAC	GCCGCGAGCTATCTTTCTTCA
HCN4	TGGACACCGCTATCAAAGTGG	CTGCCGAACATCCTTAGGGA
KCNJ2	AGCCTATGGTTGTCTGGGTCT	TGGATGCTGGTTATCTTCTGC
LIN52	CTAGTTCTCCACCCAAATGGATG	GCTGATAGGCTAGGTTCTGTAGG
LPIN1	CCAGCCCAATGGAAACCTCC	AGGTGCATAGGGATAACTTCCTG
LPL	CGAGTCGTCTTTCTCCTGATGAT	TTCTGGATTCCAATGCTTCGA
MAPK1	TACACCAACCTCTCGTACATCG	CATGTCTGAAGCGCAGTAAGATT
MKI67	ACGCCTGGTTACTATCAAAAGG	CAGACCCATTTACTTGTGTTGGA
MYC	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
MYH6	GCCCTTTGACATTCGCACTG	GGTTTCAGCAATGACCTTGCC
MYH7	TCACCAACAACCCCTACGATT	CTCCTCAGCGTCATCAATGGA
MYOCD	ACGGATGCTTTTGCCTTTGAA	AACCTGTCGAAGGGGTATCTG
ND1	CCCTAAAACCCGCCACATCT	GAGCGATGGTGAGAGCTAAGGT
NKX2-5	CACCGGCCAAGTGTGCGTCT	GCAGCGCGCACAGCTCTTTC
PPARGC1a	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTCACATCTAGTTCA
PFKM	GGTGCCCGTGTCTTCTTTGT	AAGCATCATCGAAACGCTCTC

PTEN	TGGATTCGACTTAGACTTGACCT	GGTGGGTTATGGTCTTCAAAAGG
PYGM	CCATGCCCTACGATACGCC	TAGCCACCGACATTGAAGTCC
RB1	CTCTCGTCAGGCTTGAGTTTG	GACATCTCATCTAGGTCAACTGC
RBL2 (p130)	CCACCCCTCAGATCCAGCA	CGTGTAGCTTTCGCTCATGC
REST	GCCGCACCTCAGCTTATTATG	CCGGCATCAGTTCTGCCAT
RYR2	AGAACTTACACACGCGACCTG	CATCTCTAACCGGACCATACTGC
SCN5A	TCTCTATGGCAATCCACCCCA	GAGGACATACAAGGCGTTGGT
SLC2A1 (GLUT1)	CGCTTCCTGCTCATTAACCG	ACTCTCTTCCTTCATCTCCTG
SLC2A5 (GLUT4)	GCTCATCCTTGGACGATTCC	CACCTGGGCGATCAGAATG
SLC27A1 (FATP1)	CTGCCCTTAAATGAGGCAGTC	AACAGCTTCAGAGGGCGAAG
SLC27A6 (FATP6)	TGCGTGGTGGCCTTTCTC	CAGGCGCGGATGCAATTC
SRF	CGAGATGGAGATCGGTATGGT	GGGTCTTCTTACCCGGCTTG
TBP	CCCGAAACGCCGAATATAATCC	AATCAGTGCCGTGGTTCGTG
TNNI1	CCGGAAGTCGAGAGAAAACCC	TCAATGTCGTATCGCTCCTCA
TNNI3	TTTGACCTTCGAGGCAAGTTT	CCCGGTTTTCCTTCTCGGTG
TNNT2	GGAGGAGTCCAAACCAAAGCC	TCAAAGTCCACTCTCTCTCCATC
TP53 (p53)	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC

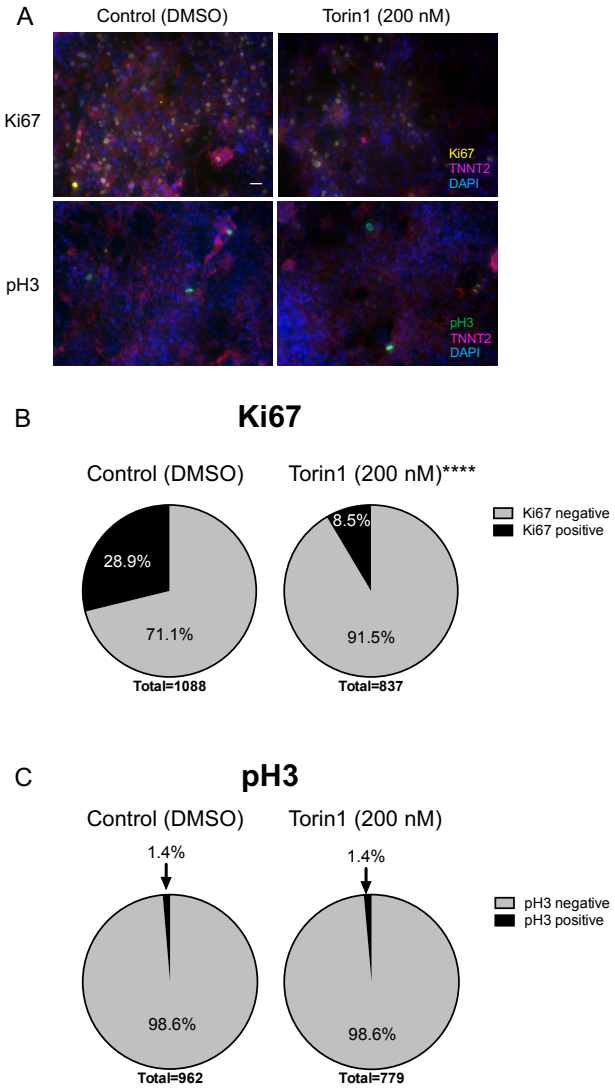
Supplemental Table 2. Primary and secondary antibodies used in study

Protein	~MW (kDa)	Assay	Primary antibody	Secondary antibody
Alpha-actinin	103	ICC	Mouse monoclonal anti-sarcomeric alpha-actinin (EA-53) (Abcam ab9465)	Goat anti-mouse IgG (H+L) 2˚ Ab, AlexaFluor 568 conjugate (ThermoFisher A-11004)
Beta-tubulin	50	Western	Rabbit polyclonal anti-beta tubulin antibody, HRP conjugate (ab21058)	n/a
DRP1	80	Western	Rabbit monoclonal anti-DRP1 (D8H5) (Cell Signaling #5391)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721019)
GAPDH	37	Western	Rabbit monoclonal anti-GAPDH (D16H11) (Cell Signaling 5174S)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721019)
F-actin		ICC	AlexaFluor 488 Phalloidin (ThermoFisher A12379)	n/a
GATA4	44	Western	Rabbit polyclonal anti-GATA4 (Abcam #ab84593)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
Ki-67		ICC	Rabbit monoclonal anti-Ki-67 (clone D3B5), AlexaFluor 488 conjugate (Cell Signaling #11882)	n/a
Kir2.1 (KCNJ2)	48	Western	Rabbit monoclonal anti-Kir2.1 (clone 2153C) (R&D Systems MAB9548)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
Kir2.1 extracellular	48	Flow cytometry, Western	Rabbit polyclonal anti-Kir2.1 (extracellular) (Alomone #APC-159)	Goat Anti-rabbit (H+L) 2˚ Ab, AlexaFluor 488 conjugate (Thermo A11034) (FACS) or Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad) (Western)
MFN1	82	Western	Rabbit monoclonal anti-MFN1 (D6E2S) (Cell Signaling #14739)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721019)
Nkx2.5	~35	Western	Rabbit monoclonal anti-NKX2.5 (E1Y8H) (Cell Signaling #8792)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
OPA1	80	Western	Rabbit monoclonal anti-OPA1 (D7C1A) (Cell Signaling #67589)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721019)
p21	21	Western	Rabbit monoclonal anti-p21 Waf1/ Cip1 (12D1) (Cell Signaling #2947)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
p53	53	Western	Mouse monoclonal anti-p53 (DO-7) (Cell Signaling #48818)	Goat anti-mouse IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721011)
p130	130	Western	Rabbit monoclonal anti-p130 (phospho T986) [EPR2389(2)] (Abcam 211928)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)

Phospho-Akt	60	Western	Rabbit polyclonal anti-phospho-Akt (Ser 473) (Cell Signaling #9271)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
Phospho-H3		ICC	Mouse mAb anti-phospho-Histone H3 (ser10) (3H10) (Millipore 05-806)	Goat anti-mouse IgG1 2˚ Ab, AlexaFluor 488 conjugate (ThermoFisher A-21121)
Phospho-p53	53	Western	Rabbit polyclonal anti-phospho-p53 (Ser15) (Cell Signaling #9284)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
Phospho-S6K	32	Western	Rabbit monoclonal anti-phospho-S6 ribosomal protein (Ser240/244) (D68F8) XP (Cell Signaling #5364)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
TFAM	24	Western	Rabbit monoclonal anti-TFAM (D5C8) (Cell Signaling #8076)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721019)
Troponin I (TNNI1)	22	Western	Rabbit polyclonal anti-troponin I type 1 (skeletal, slow) (Sigma AV42117)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad1721019)
Troponin I (TNNI3)	24	Western	Rabbit polyclonal anti-cardiac troponin I antibody (Abcam ab47003)	Goat anti-rabbit IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad)
Troponin T2 (TNNT2)	35	Western ICC	Mouse monoclonal anti-troponin T type 2 (cardiac) antibody, isoform Ab-1 (ThermoFisher MS-295-P0)	Goat anti-mouse IgG1 2˚ Ab, Goat anti-mouse IgG (H+L) 2˚ Ab, HRP conjugate (Bio-Rad 1721011) (Western) AlexaFluor 488 conjugate (ThermoFisher A-21121) (ICC)
Troponin T2 (TNNT2)	35	Flow cytometry	Alexa Fluor 647 mouse monoclonal anti-cardiac troponin T (clone 13-11) (BD Biosciences 565744)	n/a

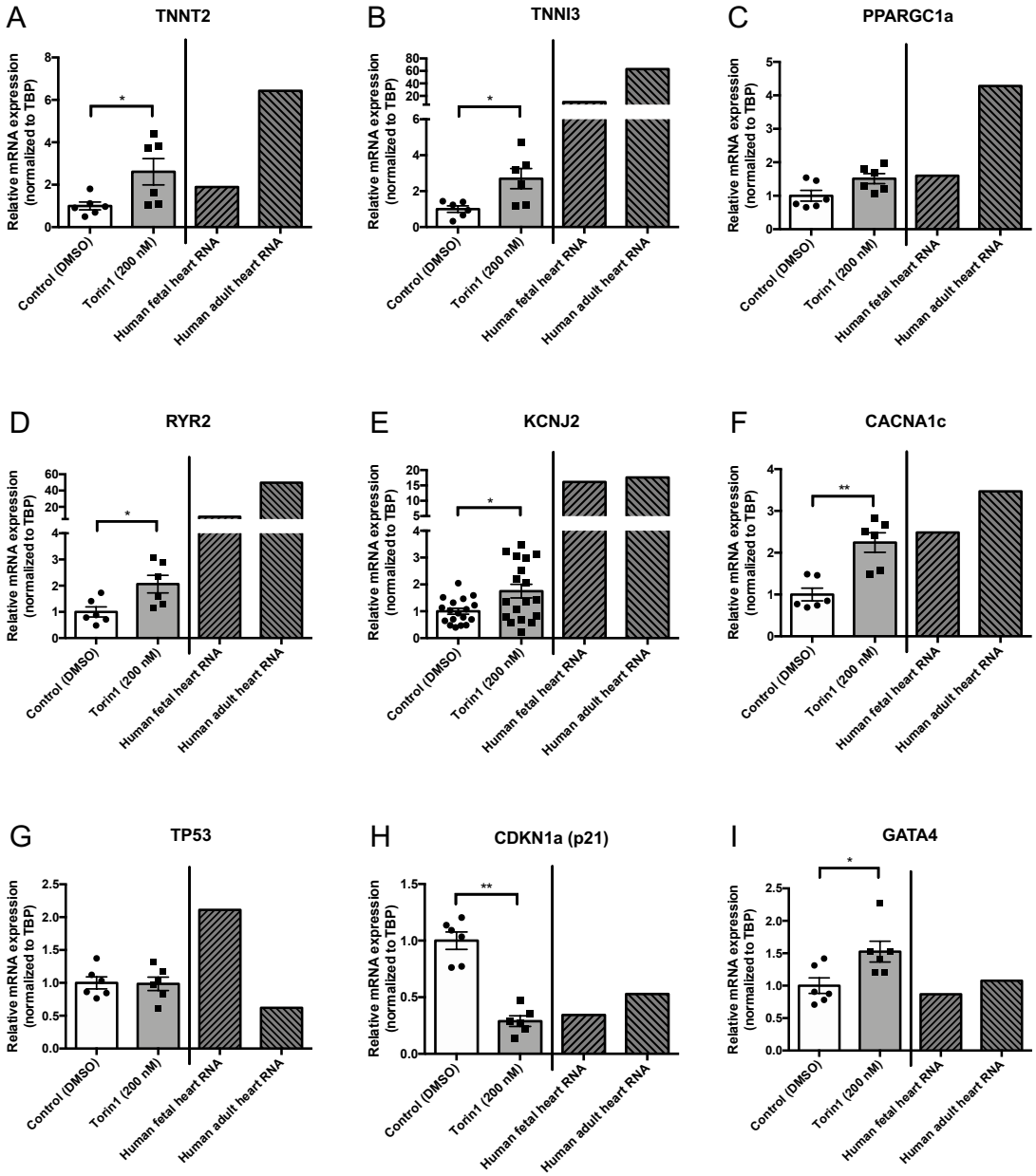
SUPPLEMENTAL FIGURES

Supplemental Figure 1



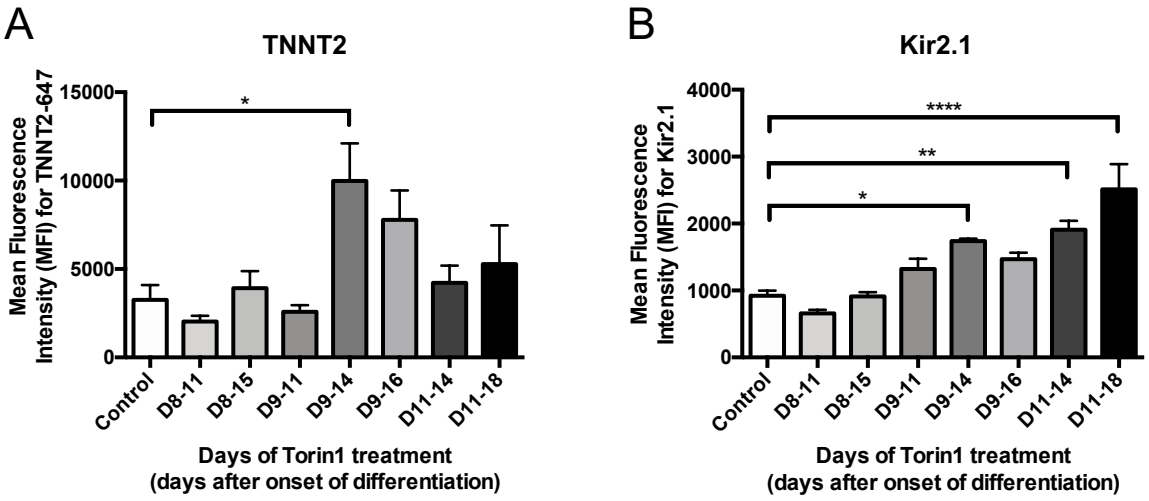
Supplemental Figure 1. Evaluation of proliferation in non-dissociated cells. Cardiomyocytes were differentiated in 12-well plates then fixed and stained in the original plates to minimize potential for selection of cells that survive dissociation. (A) Representative images of cells stained with Ki67/TNNT2/DAPI or phospho-H3/TNNT2/DAPI. Scale bar = 100 μm. Blue = DAPI, yellow = Ki67, green = pH3, magenta = TNNT2. (B) Pie charts depicting the percentage of total DAPI/TNNT+ cardiomyocytes that are Ki67+ or Ki67- in control (n=1088 cells) versus Torin1-treated cells (n=837 cells). ****p<0.0001 by chi-squared analysis. (C) Pie charts depicting the percentage of total DAPI/TNNT+ cardiomyocytes that are pH3+ or pH3- in control (n=962 cells) versus Torin1-treated cells (n=779 cells). DMSO, dimethylsulfoxide.

Supplemental Figure 2



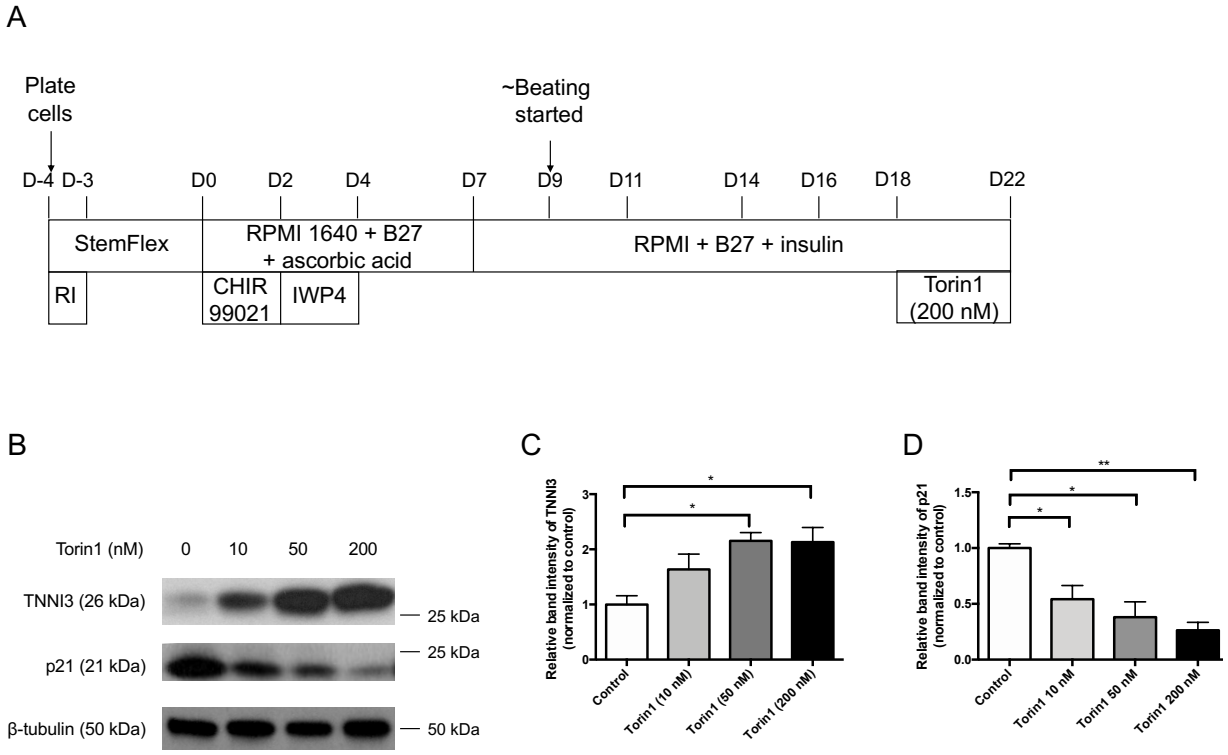
Supplemental Figure 2. qPCR analysis of selected genes (A, TNNT2; B, TNNI3; C, PPARGC1a; D, RYR2; E, KCNJ2; F, CACNA1c; G, TP53; H, CDKN1a (p21); I, GATA4) compared to benchmarking samples of commercially available human fetal and adult heart RNA. N=6-12 per group, with data combined from 2-4 independent experiments. *p<0.05, **p<0.01 by Kruskal-Wallis test, BJRiPS-CMs. Vertical line indicates that fetal and adult heart RNA were not included in statistical analysis due to lack of biological replicates (both were from single tubes purchased from commercial vendors). Error bars not shown on fetal and adult heart RNA due to lack of biological replicates. DMSO, dimethylsulfoxide; TBP, TATA-binding protein.

Supplemental Figure 3



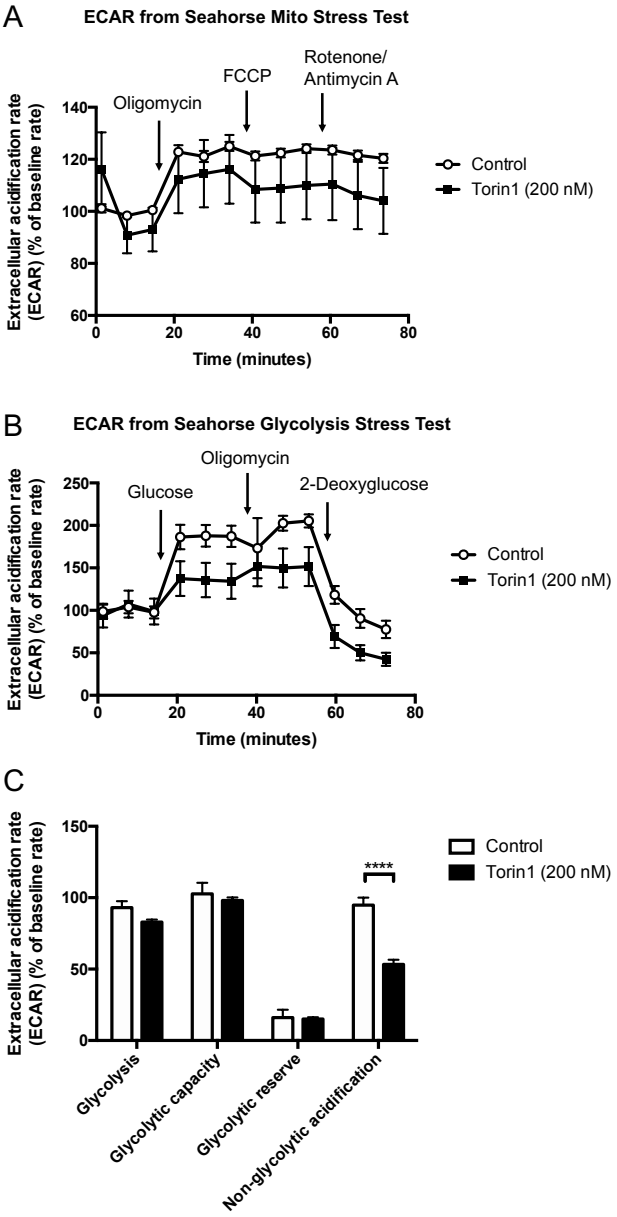
Supplemental Figure 3. Evaluation of different time periods of Torin1 treatment. (A) Mean fluorescence intensity of TNNT2-AlexaFluor647 after treatment with control (DMSO vehicle) or 200 nM Torin1 from days 8-11, 8-15, 9-11, 9-16, 11-14, or 11-18 of differentiation. n=3 per group, *p<0.05 by one-way ANOVA with Dunnett's multiple comparisons test to compare to control. (B) Mean fluorescence intensity of Kir2.1 (extracellular) after treatment with control (DMSO vehicle) or 200 nM Torin1 from days 8-11, 8-15, 9-11, 9-16, 11-14, or 11-18 of differentiation. n=3 per group *p<0.05, **p<0.01, ****p<0.0001 by one-way ANOVA with Dunnett's multiple comparisons test to compare to control.

Supplemental Figure 4



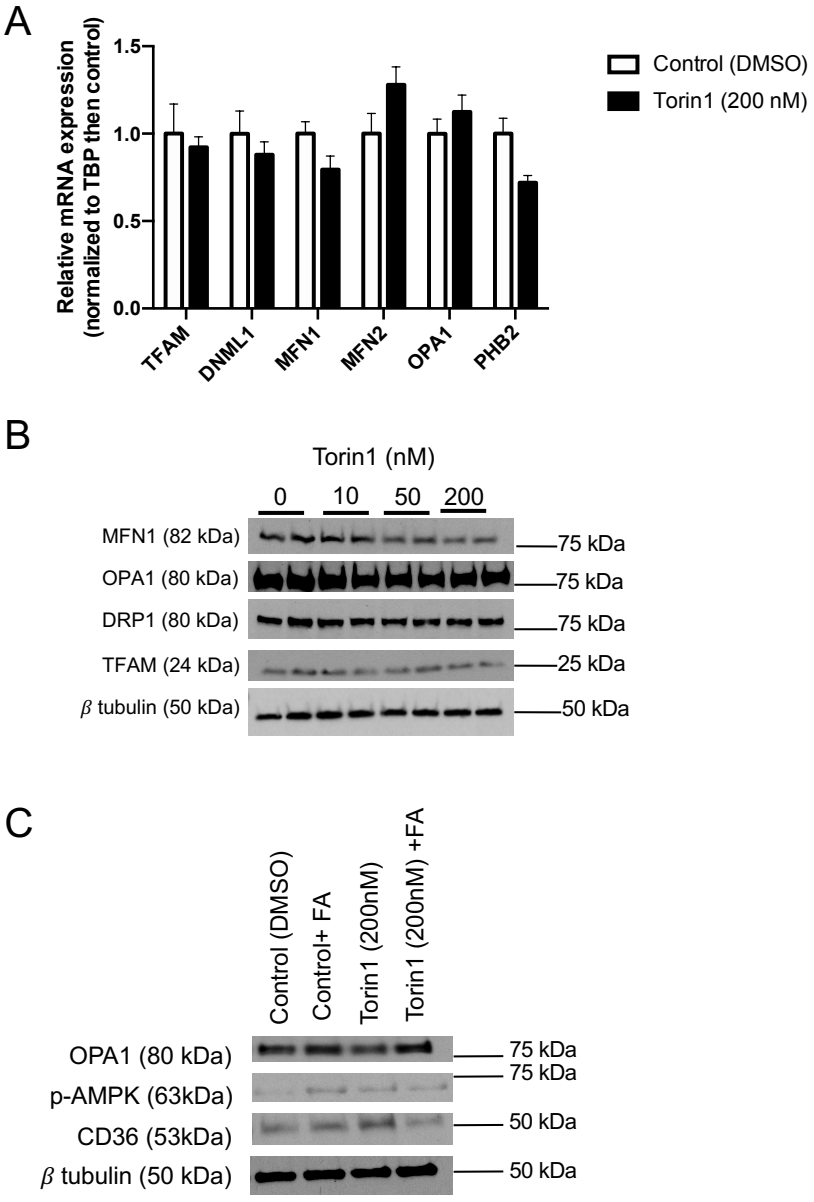
Supplemental Figure 4. Late treatment with Torin1 increases expression of TNNI3 and decreases expression of p21. (A) Schematic of protocol used for late Torin1 treatment. (B) Representative Western blot image of BJRiPS-derived cardiomyocytes treated with 0, 10, 50, or 200 nM Torin1 for 4 days prior to harvest on day 22 of differentiation then evaluated with antibodies to TNNI3, p21, or β-tubulin (loading control). (C) Relative band intensity densitometry analysis of TNNI3 bands, n=3 per group, *p<0.05 by one-way ANOVA with Tukey's multiple comparisons test. (D) Relative band intensity densitometry analysis of p21 bands, n=3 per group, *p<0.05, **p<0.01 by one-way ANOVA with Tukey's multiple comparisons test. DMSO, dimethylsulfoxide; IWP-4, inhibitor of Wnt production-4; RI, ROCK (Rho-associated, coiled coil containing protein kinase) inhibitor (Y-27632); RPMI, Roswell Park Memorial Institute 1640 medium.

Supplemental Figure 5



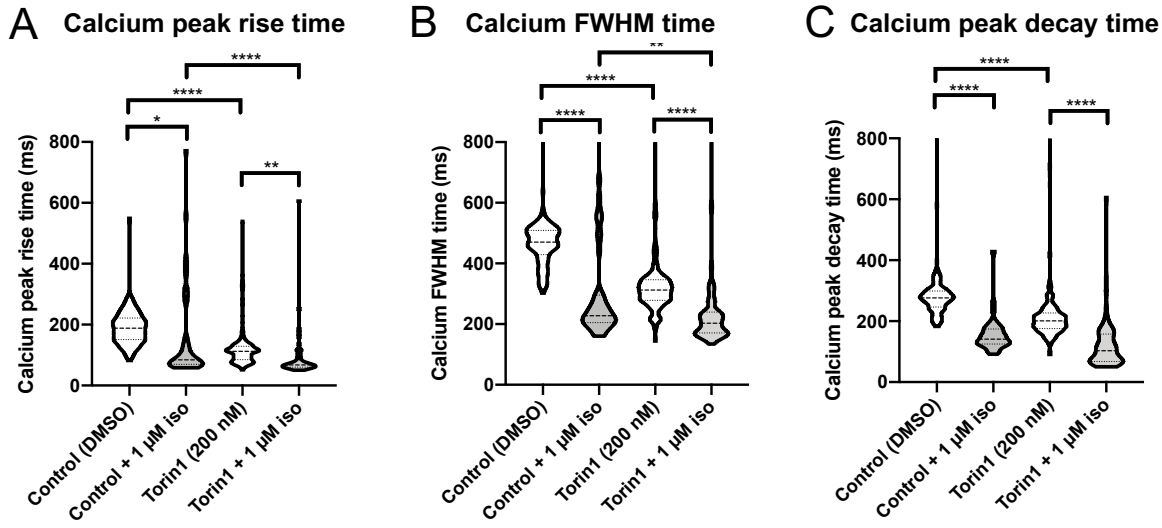
Supplemental Figure 5. Extracellular acidification rate (ECAR) in Torin1-treated cells. (A) Profile of average ECAR normalized to baseline during the Seahorse Mito Stress Test. Control (open circles, n=70 wells), Torin1 (200 nM) x 7 days (closed squares, n=69 wells), BJRiPS cell line, data combined from two independent experiments. (B) Profile of average ECAR normalized to baseline during the Seahorse Glycolysis Stress Test, n=4-6 per condition, BJRiPS cell line. (C) ECAR values for glycolysis, glycolytic capacity, glycolytic reserve and non-glycolytic acidification for control (DMSO) versus Torin1 (200 nM x 7 days) (n=4-6 per group), ****p<0.0001 by two-way ANOVA with Sidak's multiple comparisons test. FCCP, 2-[2-[4-(trifluoromethoxy)phenyl]hydrazinylidene]-propanedinitrile.

Supplemental Figure 6



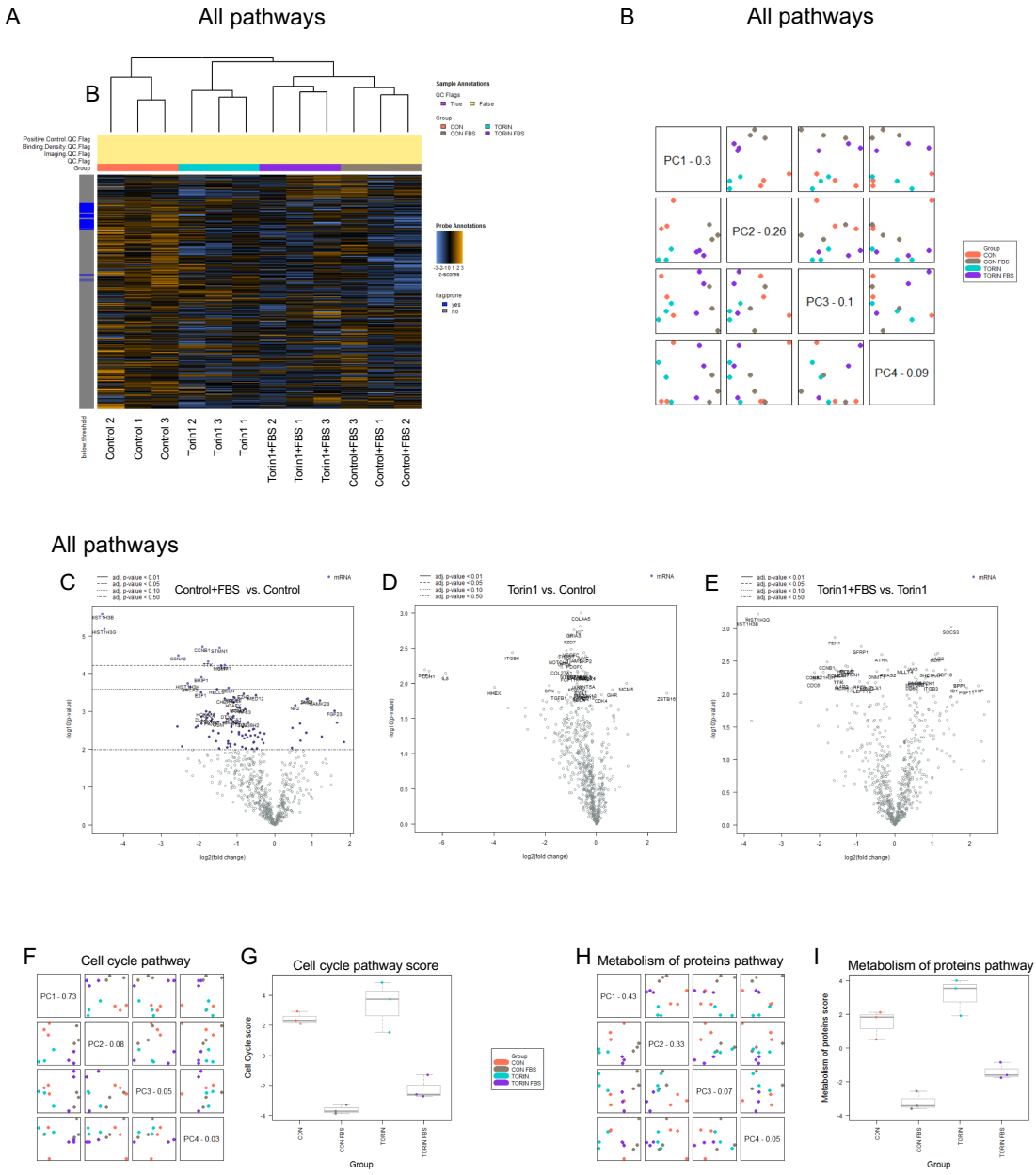
Supplemental Figure 6. Evaluation of mitochondrial gene and protein expression and effect of fatty acids. (A) qPCR analysis of selected mitochondrial genes (TFAM, DNML1, MFN1, MFN2, OPA1, PHB2). n=3 per group. (B) Representative western blot analysis of selected mitochondrial proteins (MFN1, OPA1, DRP1, TFAM1) after Torin1 treatment for 7 days starting ~2 days after onset of beating, β-tubulin depicted as loading control, Gibco iPS-CM. (C) Representative of western blot analysis of selected metabolism-associated proteins (OPA1, phosphor-AMPK, CD36) after Torin1 treatment +/- fatty acids (chemically defined lipid concentrate diluted 1:100 (Gibco) for 7 days starting ~2 days after onset of beating, β-tubulin depicted as loading control, BJRiPS-CM. DMSO, dimethylsulfoxide; TBP, TATA-binding protein.

Supplemental Figure 7



Supplemental Figure 7. Calcium transients analysis using the Vala Kinetic Image Cytometer. Fluo-4 AM dye was used to evaluate calcium handing. Isoproterenol (1 μM, "iso") was added to some wells to evaluate isoproterenol responsiveness. (A) Calcium peak rise time. (B) Calcium full width half maximum (FWHM, width of the profile at which the calcum amplitude is half of its maximum) time. (C) Calcium peak decay time. Control n=129 cells, control + 1 μM iso n=41 cells, Torin1 n=274 cells, Torin1 + 1 μM iso n=65 cells. *p<0.05, **p<0.01, ****p<0.0001, by one-way ANOVA with Sidak's multiple comparisons test.

Supplemental Figure 8



Supplemental Figure 8. NanoString gene expression analysis from PanCancer Pathways Panel comparing cells treated with or without Torin1 followed by treatment with or without 10% fetal bovine serum (FBS). (A) Unsupervised hierarchical clustering of all genes. (B) Principal component analysis of all genes. (C-E) Volcano plots showing differential gene expression analysis of all genes (C, Control + FBS versus Control; D, Control versus Torin1; E, Torin1 + FBS versus Torin1). (F) Principal component analysis of cell cycle pathway. (G) Cell cycle pathway score as determined by nSolver software analysis. (H) Principal component analysis of metabolism of proteins pathway. (I) Metabolism of proteins pathway score as determined by nSolver software analysis.